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GRANT NUMBER: DAMD17-94-J-4031

TITLE: Characterization of Ligand-induced Endocytosis of EGF-receptors

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REPORT DATE: June 1996

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

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DTIC QUALITY INSPECTED 3

19961002 071

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	June 1996	Annual (9 May 95 - 8 May 96)

4. TITLE AND SUBTITLE
Characterization of Ligand-induced Endocytosis of EGF-receptors

5. FUNDING NUMBERS
DAMD17-94-J-4031

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

We have made progress this year in engineering and using a cell line that is conditionally defective in receptor-mediated endocytosis to directly assess the role of endocytosis in controlling cellular reponses to activated EGF-R. We find, not surprisingly, that cells defective in endocytosis show a stronger proliferative response to EGF than wt cells. Surprisingly, we find that some EGF-R signaling pathways, specifically the MAP kinase activation and EGF-R phosphorylation itself are reduced in mutant cells relative to wt cells. Thus, endocytosis plays a role in specifying EGF-R signaling events, not just in attenuating them. Given the importance of endocytosis in EGF-R signaling our discovery, that Rho-family GTPases which are activated in response to growth factors are negative regulators of receptor-mediated endocytosis, may have important implications for control of receptor signalling. Finally we have begun to examine the role of eps15, a tyrosine kinase substrate of the EGF-R which binds constitutively to the AP2 endocytic coat proteins, on endocytosis of EGF-R using our *in vitro* assay.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast cancer, EGF-Receptor, Signal Transduction, Endocytosis, Erb2 Oncogene, Down-Regulation, Tyrosine Kinase	24
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

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[signature] 6/6/96

PI - Signature Date

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(5) INTRODUCTION

Peptide growth factors such as EGF bind to receptor tyrosine kinases (for example the EGF-receptor) on the cell surface to trigger signalling events leading to cell proliferation and differentiation. Ligand binding also triggers the rapid recruitment of activated receptor tyrosine kinases into coated pits, their internalization via coated vesicles and their delivery to the endosomal-lysosomal compartments for degradation. Together, these triggered membrane trafficking events lead to receptor down-regulation which is believed essential for the attenuation of signalling events. Over the past three years and continuing under the auspices of this Fellowship, we have developed a cell-free system which faithfully reconstitutes the EGF-dependent recruitment of activated EGF-R into coated pits (Lamaze et al., 1993). Last year we established that this recruitment required EGF-R kinase activity and the soluble constitutively active EGF-R kinase domains could restore ligand-induced recruitment of kinase-deficient receptors *in trans.* (Lamaze and Schmid, 1995) In addition, we obtained baculovirus expression constructs of the core EGF-R kinase domain and optimized its expression and purification. Finally, we showed that a GST-fusion protein encoding the C-terminal regulatory domain of the EGF-R and its internalization motifs, was a potent and selective inhibitor of EGF-R recruitment *in vitro.* These findings marked the successful completion of Task 1 of the original Statement of Work.

We originally proposed to use this functional assay to identify and purify the tyrosine kinase substrate(s) required for ligand induced recruitment of EGF-R into coated pits and to mechanistically characterize the molecular events involved. We have struggled, unsuccessfully, with this goal for the past year. Our technical difficulties and the attempts we are making to circumvent them will be detailed in the following section. In the meantime, we have begun, more successfully, to explore three other aspects of the regulation of receptor-mediated endocytosis as these will also provide important insight into how these processes control cellular responses to proliferative signals. First, we have begun to directly test our basic experimental tenet, that endocytosis is critical to regulating EGF-R signaling, by examining the responses to EGF in cells conditionally defective in receptor-mediated endocytosis.

The second new area of investigation is our response to the recent finding by N. Bensussan and colleagues that eps15, a tyrosine kinase substrate of the EGF-R is constitutively bound to the AP2 constituents of the endocytic clathrin coat (Benmerah et al., 1995). Based on these findings it has been proposed that eps15 may function coordinately with AP2 molecules as adaptors involved in EGF-R recruitment into coated pits. Therefore, we have initiated a collaboration with the Bensussan group to test the role of eps15 in endocytosis of the EGF-R. The initial results from this collaboration are described in the following section.

Finally, we have discovered that receptor-mediated endocytosis of both EGF-receptors and transferrin-receptors can be negatively regulated by members of the Rho family small GTPases. Rho and Rac are activated in response to a number of growth factors and they trigger the earliest and most dramatic cellular responses to stimulation by growth factors or other mitogens, namely, pinocytosis and membrane ruffling (Ridley, 1994, Hall, 1994). Our finding that Rho and Rac can act as negative regulators of

receptor-mediated endocytosis suggests that they may play an important role in prolonging surface signalling events by activated receptor tyrosine kinases. These results are reported in the attached manuscript which is *in press* in ***Nature*** . Therefore they will only briefly be described in the following section.

(6) BODY

a) Identification of kinase substrate(s) required for the recruitment of activated EGF-R into coated pits.

While this goal remains a top priority, we have encountered a number of technical difficulties which we are endeavoring to overcome. These difficulties and the approaches we are using to overcome them are as follows. 1) The core EGF-R kinase domain, as expressed and purified from baculovirus-infected SF9 cells is functionally unstable. While the cause of this instability is not known (other than to rule out proteolysis), we believe that the protein is susceptible to irreversible aggregation. Further, we have found that buffer conditions which stabilize activity, namely the inclusion of DTT, small amounts of detergent and glycerol are inhibitory to our assay system. We continue to screen buffers for reproducible purification of active kinase and for long-term storage. We also plan to switch to a new expression system using TN5 insect cells which, in other work ongoing in the lab, has proven to yield much higher levels of expression. We think that some of our stability problems can be overcome by maintaining higher protein concentrations which will be aided by higher levels of expression. We have also conducted careful titrations of buffer additives into our functional assay to determine levels of tolerance. At higher protein concentrations, we can add less buffer into our assays while maintaining levels of kinase needed for activity. 2) The efficiency of EGF-R endocytosis both *in vivo* and *in vitro* has decreased over time and we have determined that this is due to heterogeneity in our transformed cell lines after long-term culture. We have therefore contacted Dr. Steven Wiley (U. of Utah) who originally developed and characterized the B82L cell transformants expressing wt and mutant EGF-R. He has agreed to send us vials from early freezings once he has confirmed their properties. 3) We have been attempting to deplete cytosols of EGF-R recruiting activity using the GST-regulatory domain fusion protein that is inhibitory in our assay. We have found that passing cytosol over control GST-Sepharose beads can severely inhibit activity and so we are currently optimizing our depletion conditions to obtain specific results. The optimizations include the ratio of beads to cytosol and the experimental conditions, for example batch vs. column, time of elution, etc. We hope to overcome these difficulties in order to successfully complete this most important objective.

b) Role of endocytosis in EGF-receptor signaling

We have been using the HeLa cells conditionally defective in endocytosis to explore the role of endocytosis in EGF-R signaling. We have generated stable HeLa cell lines conditionally defective in receptor-mediated endocytosis by inducible expression of dominant-negative mutants of dynamin, a GTPase required for coated vesicle

budding. In previous studies we have established that expression of the K44A mutant of dynamin, which is defective in GTP binding and hydrolysis, potently and selectively inhibits receptor-mediated endocytosis: no other cellular functions appear affected (Damke et al., 1994). We find, that cells expressing mutant dynamin cells show significantly stronger proliferative responses to EGF confirming previous findings by others that endocytosis attenuates EGF-R signaling (Figure 1).

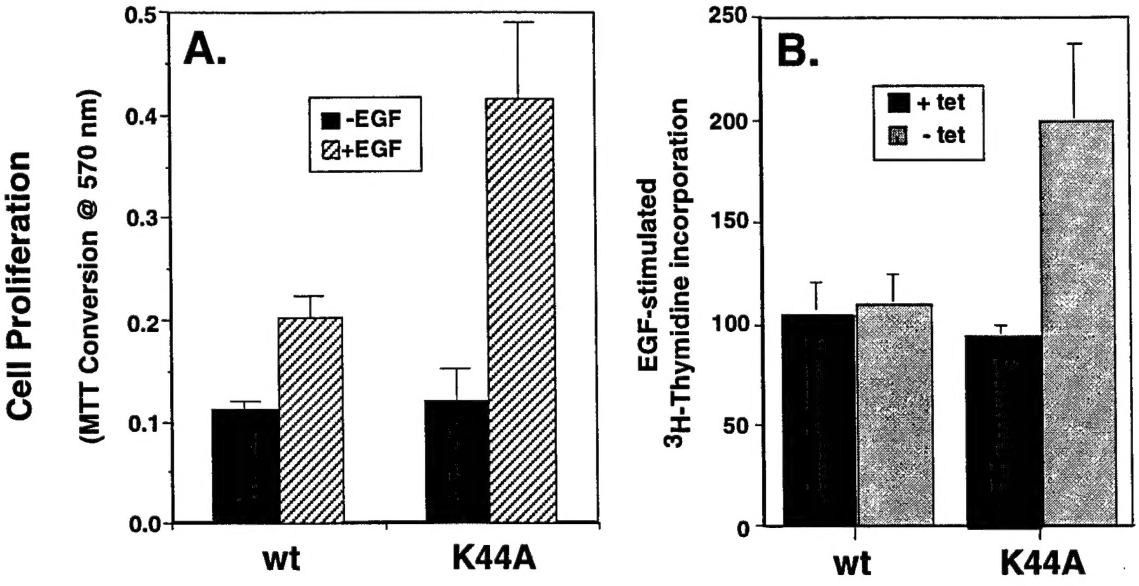

Figure 1: Cells conditionally defective in endocytosis show a stronger proliferative response to EGF. HeLa cells were stably transformed with either wt dynamin or the K44A mutant of dynamin defective in GTP binding and hydrolysis under the control of the tetracycline (tet) regulatable promoter. In the absence of tet, expression is induced and receptor-mediated endocytosis is potently and specifically inhibited in cells expressing K44A mutant dynamin but not wt dynamin (Damke et al., 1994). These cells were starved for 24 hr in low serum-containing media in the presence (Panel A) or absence (as indicated in Panel B) of tet to induce dynamin expression. EGF was then added (4 nM) for a further 24 hr and cell proliferation was measured either using a quantitative colorimetric assay for cell number (Panel A) or by ^{3}H-thymidine incorporation (Panel B) using standard assay conditions. Panel A shows that the number of K44A cells increase ~two-fold more in response to EGF than wt cells. Panel B shows that K44A cells grown in the presence of tet to suppress mutant dynamin expression exhibit the same level of EGF-dependent induction of ^{3}H-thymidine incorporation as wt cells (grown in the presence or absence of tet), but in the absence of tet, under conditions that induce mutant dynamin expression, the K44A cells again show an ~2-fold increase in the proliferative response to EGF, compared to wt cells.

Interestingly, as shown in Figure 2, we have found that while some cellular responses to EGF are upregulated in K44A cells (eg. EGF-dependent tyrosine phosphorylation in general and PLC$_\gamma$ and SHC phosphorylation in particular) others are reduced (eg. ERK tyrosine phosphorylation and correspondingly MAP kinase activation (not shown) and EGF-R phosphorylation). These findings, which are summarized in Figure 2, imply that endocytosis may help define EGF-R signalling and that endocytosis may be an important modulator of signaling events. A manuscript describing these results is currently in preparation (Vieira, A., C. Lamaze and S.L. Schmid, *manuscript in preparation*)

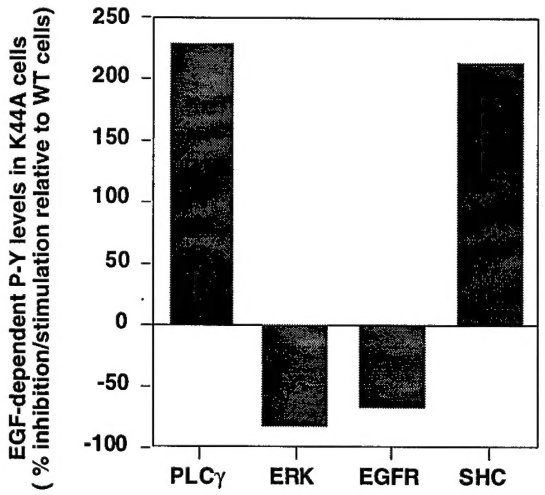

Figure 2: Differential EGF-R signaling in cells conditionally defective in receptor mediated endocytosis. HeLa cells expressing either wt or K44A mutant dynamin were serum-starved overnight and then treated with EGF (4 nM) for 20 min. Cell lysates were prepared in the presence of tyrosine phosphatase inhibitors and immunoprecipitated with the anti-phosphotyrosine specific antibody, 4G10. Immunoprecipitates were subjected to SDS-PAGE, transferred to nitrocellulose and blotted for the signaling molecules indicated, i.e., PLCγ, the ERK MAP kinase, the EGF-R or SHC. Data is expressed as the percent inhibition or stimulation of phosphotyrosine incorporated into these proteins in K44A cells relative to wt cells. As can be seen, while PLCγ and SHC showed increased levels of phosphotryosine incorporation, consistent with the increased proliferative response of K44A cells to EGF, both ERK and EGF-R showed decreased levels of phosphotyrosine incorporation. These data suggest that internalization of the EGF-R plays a role in modulating the nature of EGF-R signaling distinct from its role in attenuating the extent of EGF-R signaling.

c) Role of eps15 in EGF-R endocytosis

Eps15 was originally cloned in an expression screen for EGF-R tyrosine kinase substrates. Overexpression of eps15 induces a transformed phenotype in normal human fibroblasts (Fazioli et al., 1993). The N-terminal of eps15, the so-called domain-1 was found to have sequence identity with End3p, a yeast protein involved in endocytosis (Bénédetti et al., 1994). Domain-I also contains two Ef-hand repeats suggesting a possible role of Ca^{2+}in regulating its function. The C-terminal proline-rich domain of eps15, the so-called domain-III, has 13-15 DPF repeats, a motif common to methyl-transferases and is therefore thought to be the 'effector' domain. Recent results from Bensussan and colleagues (personal communication) have established that AP2 interactions are also mediated through domain 3. The middle domain-II contains heptad coiled-coil motifs.

We have begun a multi-pronged approach to studying eps15 function in receptor-mediated endocytosis *in vitro*.

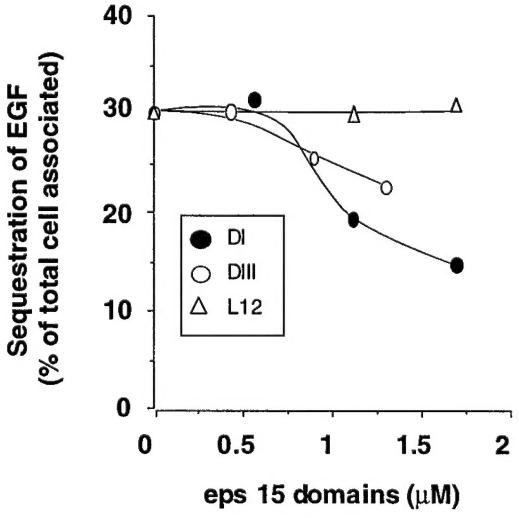

Fig. 3: Effect of eps 15 domains on endocytosis of EGF *in vitro*. Perforated A431 cells were incubated for 30 min at 37°C with K562 cytosol, an ATP regenerating system and B-EGF. Incubations were stopped on ice and EGF receptor-mediated endocytosis was determined by measuring the sequestration of biotinylated EGF (B-EGF) from exogenously added avidin as described (Lamaze et al., 1993). The data show the effect of increasing concentrations of GST-eps 15 fusion proteins on ATP and cytosol-dependent EGF sequestration. GST-eps 15 fusion proteins have been characterized elsewhere (Benmerah et al., 1996). DI (●) presents the 3 EH domains (aa 1-315), DIII (○) codes for the DPF repeats (aa 529-896). L12 (Δ) corresponds to aa 667-697, a region of the DIII which does not immunoprecipitate AP-2 complexes *in vivo* (Benmerah et al.,1995).

First, we have examined whether the individual domains of eps15, expressed as GST-fusion proteins in E. Coli effect endocytosis *in vitro*. The preliminary data shown in Figure 3 suggests that the two functional domains DI and DIII on their own inhibit endocytosis of EGF. We presume that this inhibition is due to nonproductive interaction with upstream and/or downstream partners of eps15. Secondly, we are using antibodies directed against eps15 to test whether it is required for EGF-R endocytosis *in vitro*. The preliminary data in Figure 4 shows that cytosol immunodepleted of eps15 is incapable of supporting EGF internalization *in vitro*.

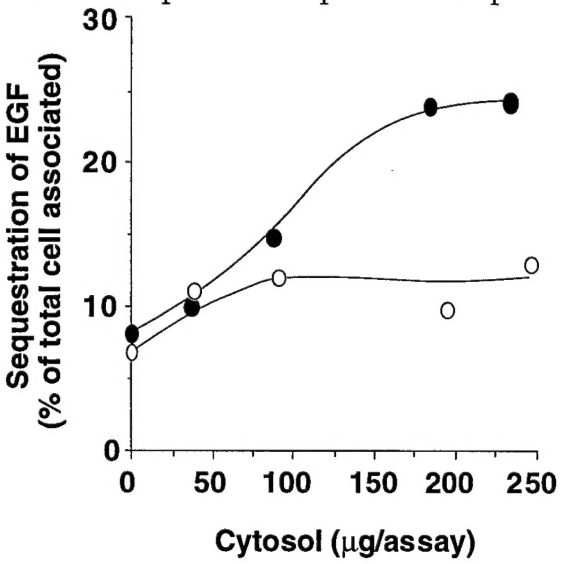

Figure 4: Eps15 immunodepleted cytosol does not support efficient sequestration of activated EGF receptors *in vitro*. 6G4, a monoclonal antibody against Eps15 (Benmerah et al., 1995) was used to immunodeplete cytosol from K562 human erythroleukemic cells. 6G4 antibody (100μg/ml) was incubated with cytosol overnight at 4°C and retrieved by incubation with protein A sepharose beads for two hours at 4°C. The data show the amount of EGF sequestration supported by increasing concentrations of K562 cytosol treated with(○) or without (●) anti-Eps 15 antibody. EGF sequestration was quantitated as described in Figure 3.

Third, since yeast cytosol supports endocytosis *in vitro* using perforated mammalian cells, we are testing the ability of cytosols from wt and end3-deficient yeast to support endocytosis *in vitro*. We may be able to complement the end3 defect with eps15.

We are excited by these findings as they suggest that eps15, a major substrate for the EGF-R *in vivo* may be important in regulating endocytosis. Interestingly, eps15 binds to AP2 molecules constitutively and we are observing effects on both the constitutive endocytosis of Tfn-R as well as the ligand-induced endocytosis of EGF-R. Thus, it will be important to determine how tyrosine phosphorylation of eps15 by the EGF-R affects its function. We suspect that eps15 may not be the only tyrosine kinase substrate required for EGF-R endocytosis.

d) Regulation of receptor-mediated endocytosis by Rho and Rac

Rho and Rac are small *ras*-related GTPases which are activated in response to peptide growth factors. In collaboration with Dr. Gary Bokoch at The Scripps Research Institute, we recently discovered that receptor-mediated endocytosis of Tfn was potently inhibited in cells transiently transfected with activating mutants of Rho and Rac but not in cells expressing wild type or inactivated mutants of these proteins. We were able to reconstitute Rho and Rac-regulation of receptor-mediated endocytosis in our cell-free system and to further establish that Rho and Rac inhibited late events in coated vesicle budding. The data in Figure 5A shows that RhoA and Rac inhibited EGF-R endocytosis

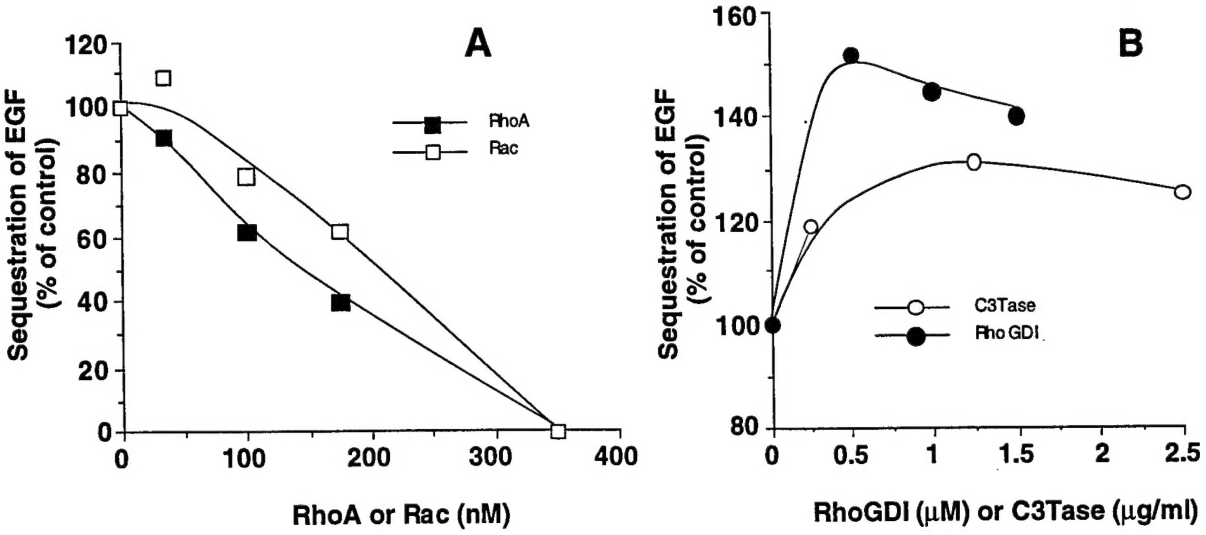

Figure 5: Rho and Rac regulate the sequestration of EGF receptors *in vitro*. Perforated A431 cells were incubated as described in Figure 3. (**A**) Assays were performed in the presence of increasing concentrations of wild type Rac (□) or Rho (■) prepared from baculovirus-infected SF9 cells as described (Xu et al.,1994). (**B**) Assays were performed in the presence of increasing concentrations of purified recombinant RhoGDI (○) or recombinant GST-C3 transferase (●) (obtained from Dr. Simon Dillon, Tufts University) expressed and purified from E. Coli.

in vitro . In a complementary manner, inactivation of endogenous Rho (and Rac) by RhoGDI (which binds Rho-family GTPases and locks them in their GDP-bound inactive conformation) or by ADP-ribosylation of Rho with the C3 botulinum exoenzyme stimulated coated vesicle budding *in vitro* (Figure 5B).

These results (see Lamaze et al. 1996) suggested that Rho and Rac serve as negative regulators of endocytosis. The physiological significance of these findings and the mechanism by which Rho and Rac exert their effects are being explored. However, it is intriguing to speculate that EGF-R activation of Rho and/or Rac might be a positive feed-back to surface signaling by the EGF-R as it would be predicted to increase its surface residency by negatively regulating endocytosis. This model needs to be tested.

(7) CONCLUSIONS

In the past year we have continued to explore the mechanisms regulating endocytosis of activated EGF-R and the functional consequences of this regulation. While we have encountered unexpected technical difficulties in our originally proposed biochemical approach towards identifying factors required ligand-induced EGF-R into coated pits, we have been quite successful in identifying new factors that regulate endocytosis and in defining the physiological role of endocytosis in receptor signalling. In addition, we have taken advantage of advances made by others and have established collaborations to explore the role of eps15, a major EGF-R tyrosine kinase substrate in endocytosis. It is likely that this protein represents one of the factors required for ligand-induced endocytosis of EGF-R.

(8) REFERENCES

Bénédetti, H., S. Rahts, F. Crausaz and H.U. Riezman (1994) The END3 gene encodes a protein that is required for the internalization step of endocytosis and for actin cytoskeleton organization in yeast. Mol Biol. Cell 5:1023-1037

Benmerah, A., J. Gagnon, B. Bègue, B. Mégarbané, A. Dautry-Varsat and N. Cerf-Bensussan (1995) The tyrosine kinase substrate eps15 is constitutively associated with the plasma membrane adaptor AP-2 J. Cell Biol. 131:1831-1838

Benmerah, A., B. Bègue, A. Dautry-Varsat, N. Cerf-Bensussan (1996) The ear of α-adaptin interacts with the COOH terminal domain of the Eps15 protein. J. Biol. Chem. 271 *in press*

Damke, H., Baba, T., Warnock, D.E. and Schmid, S.L. (1994) Induction of Mutant Dynamin Specifically Blocks Endocytic Coated Vesicle Formation. J. Cell Biol., 127:915-934

Fazioli, F., L. Minichiello, B. Matoskova, W. T. Wong and P. Paolo di Fiore (1993) eps15, a novel tyrosine kinase substrate, exhibits transforming activity. Mol. Cell. Biol. 13:5814-5828

Hall, A. Small GTP-binding proteins and the regulation of the actin cytoskeleton. *Ann. Rev. Cell Biol.* 10:31-54 (1994)

Lamaze, C. and Schmid, S.L. (1995) Recruitment of Epidermal Growth Factor Receptors into Coated Pits Requires Their Activated Tyrosine Kinase. J. Cell Biol. 129:47-54

Lamaze, C., Baba, T., Redelmeier, T.E. and S.L. Schmid (1993) Recruitment of EGF- and Transferrin Receptors into Coated Pits *in vitro*: Differing Biochemical Requirements. Mol. Biol. Cell 4: 715-727.

Lamaze, C., T.-H Chuang, L.J. Terlecky, G. M. Bokoch and S. L.Schmid (1996) Regulation of transferrin receptor-mediated endocytosis by rho and rac. Nature *in press*

Ridley, A. J. Membrane ruffling and signal transduction. *BioEssays* 16:321-327 (1994)

APPENDIX
DAMD17-94-J-4031

MS: S02364

Revised May 22, 1996

Regulation of Receptor-mediated Endocytosis by Rho and Rac

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Pinocytosis and membrane ruffling are among the earliest and most dramatic cellular responses to stimulation by growth factors or other mitogens (1). A regulatory role for the small ras-related G-proteins, Rho and Rac in membrane ruffling has been clearly established (1-3). More recently, activated Rho has been shown to stimulate pinocytosis when microinjected into *Xenopus* oocytes (4). In contrast to these well-established effects of Rho and Rac on plasma membrane morphology and bulk pinocytosis, there has been no evidence for their involvement in the regulation of receptor-mediated endocytosis via clathrin coated pits. Here we show that activated Rho and Rac inhibit transferrin-receptor-mediated endocytosis when expressed in intact cells. Furthermore, we have reconstituted these effects in a cell-free system and established that Rho and Rac can regulate clathrin coated vesicle formation.

Receptor-mediated endocytosis of TexasRed-conjugated transferrin (Tfn) was measured in transiently transfected cells expressing wild-type or mutant forms of Rac and Rho. Intracellular accumulation of Tfn was potently (>95% of transfected cells) inhibited in HeLa cells transiently expressing an activated, GTP-bound Rac mutant (Q61L) (Figure 1, e and f) but not affected (<5%) in cells expressing either wt Rac (Figure 1, a and b) or an inactivated, GDP-bound mutant, Rac^{T17N} (Figure 1, c and d). Similar results were obtained with activated Rho^{Q63L} (Figure 1, g and h), but not with either a GDP-Rho mutant (T19N) or wt Rho (data not shown).

Inhibition of intracellular Tfn accumulation in transiently transfected cells could reflect Rac and Rho effects at any stage of the internalization, sorting and recycling pathway taken by transferrin receptors. While we cannot rule out effects of Rho-family GTPases on later endocytic membrane trafficking events, their role in internalization was further supported by our finding that cells expressing the activating mutant of Rac (Q61L), but not wt or Rac^{T17N}, could not be infected with Vesicular Stomatitis Virus (data not shown). Nevertheless, to confirm and further investigate their early role in endocytosis, we tested the effects of Rho and Rac in a cell-free system that reconstitutes the internalization of receptor-bound Tfn via clathrin coated pits (5,6). Rac and Rho were both potent inhibitors (half-

maximal inhibition at ~25-30 nM) of Tfn receptor-mediated endocytosis in perforated A431 cells (Figure 2*a*). The inhibitory effects of Rac and Rho required their post-translational processing, since E. coli-expressed proteins had no effect (data not shown.)

Inhibition by the wt GTPases *in vitro,* could reflect quantitative differences in the amount of protein assayed or differences in GAP activity *in vivo* and *in vitro,* We were also unable to discern differences in the ability of Rac or Rho to inhibit endocytosis *in vitro* when preloaded either with GDPβS or GTPγS, presumably because these GTPases are able to rapidly exchange nucleotides in cell lysates (7). Therefore, to confirm the specificity of inhibition by activated forms of Rho and Rac as seen *in vivo,* assays were performed in the presence of excess RhoGDI. RhoGDI forms a stoichiometric complex with all members of the Rho-GTPase family, inhibits GDP-GTP exchange and locks them in their inactivated GDP-bound states (8). Inclusion of RhoGDI substantially blocked the inhibitory effects of Rho and Rac (Figure 2*b*). These experiments established that the GTP-bound forms of Rho and Rac inhibit an early event in receptor-mediated endocytosis both *in vivo* and *in vitro.*

To determine whether Rho or Rac activities are constitutively required for receptor-mediated endocytosis, perforated A431 cells were incubated in the presence of RhoGDI alone. Addition of either RhoGDI or its homologue Ly-GDI/D4 (9,10) stimulated receptor-mediated endocytosis of Tfn (Figure 2*c*). This result argues that Rho and Rac are not required for endocytosis and instead suggests that endogenous Rho and Rac might be negative regulators of endocytosis under the conditions of our assay. Consistent with this, recombinant C3-transferase, a bacterial toxin that specifically inactivates Rho through ADP-ribosylation of Asn41 (11) also stimulated Tfn-receptor-mediated endocytosis in perforated A431 cells (Figure 2*d*). Thus, activation and inactivation of Rho have opposing effects on Tfn-sequestration *in vitro.* In all cases, receptor-mediated endocytosis of EGF was similarly affected (data not shown) indicating that Rho and Rac were general regulators of endocytosis via clathrin coated vesicles. It should be noted that the effects seen with Rho-family GTPases were selective since neither rab-family GTPases, Rab GDI nor Arf1 GTPase had any effect on endocytosis *in vitro* (L. Terlecky and S. Schmid, unpublished observations).

In some cases, Rac has been shown to act through Rho in regulating downstream events (1, 12). Thus, the Rac effects could either be mediated directly through a common downstream effector of both GTPases or, indirectly, through Rho. However, we found that C3 transferase, which specifically inactivates endogenous Rho, could not block inhibition by Rac (Figure 2*e*). Thus, Rac inhibition appears to be independent of Rho.

The overall process of coated vesicle formation *in vitro* can be dissected into early events leading to the sequestration of receptor-bound Tfn into constricted coated pits and late events involved in coated vesicle budding (6). Coated vesicle budding is selectively detected when Tfn, biotinylated via a cleavable disulfide bond, is internalized into sealed membrane vesicles and becomes inaccessible to the small membrane impermeant reducing agent, β-mercaptoethane sulfonic acid (MesNa). Using this assay, we found that both the rate and extent of coated vesicle budding were severely inhibited in the presence of Rho (or Rac, data not shown) and, in a reciprocal manner, were enhanced by the addition of RhoGDI (Figure 3).

Our data suggests that the effects of Rho and Rac are mediated by a common downstream effector. Since both of these small GTPases control, directly or indirectly, assembly of the actin cytoskeleton (2,3), we examined whether their effects on endocytosis might be related to effects on actin by testing whether cytochalasin D which inhibits actin assembly, or phalloidin which stabilizes actin filaments, would antagonize or synergize with Rac and Rho. As can be seen (Figure 4), neither cytochalasin D (when present at 1-10 μg/ml) nor phalloidin (when present at 0.5-10 μM) inhibited receptor-mediated endocytosis of Tfn on their own. More importantly, neither altered the inhibitory effects of Rac and Rho on Tfn endocytosis. Cytochalasin D-treatment similarly did not affect the inhibition of Tfn accumulation seen in cells transiently transfected with either mutant Rac or Rho (data not shown). These results argue against the simple hypothesis that the Rho and Rac effects are due to triggered assembly of the actin cytoskeleton. However, the effects of cytochalasin D on cortical actin assembly/disassembly are poorly characterized and we cannot, therefore, rule out a role for this subpopulation of actin filaments in regulating endocytosis.

The common downstream effector(s) responsible for the regulation of clathrin-mediated endocytosis by Rho and Rac have not been identified. Intriguing candidates include phospholipase D (PLD) (13, 14), phosphoinositol 4-phosphate 5 kinase (PI 5-Kinase) (15-17) and PI 3-kinase (17, 18) which has also been identified as an upstream regulator of Rac required for growth factor-stimulated membrane ruffling (19-21). However, wortmannin, a potent inhibitor of PI 3-kinase, has no effect either on Tfn- or EGF endocytosis in intact or perforated cells (21, 22; CL and SLS, unpublished observations). It remains possible that activation of PLD and/or PI 5-kinase could directly control clathrin coated vesicle formation, consistent with recent proposals that site-directed lipid modifications could trigger the recruitment of components required to initiate vesicle budding (23, 24). Alternatively, activated Rho or Rac could indirectly affect coated vesicle formation by mistargeting, to multiple intracellular sites, the assembly of limiting components essential for coated vesicle budding. In this context it is interesting to note that GTPγS treatment of cells results in the mistargeting of AP2 adaptor complexes from coated pits on the plasma membrane to the endosomal compartment (25). Receptor-mediated endocytosis *in vitro*, only the second cell-free assay system in which the effects of Rho and Rac have been reconstituted, should provide a functional means for identifying the relevant downstream effectors.

In summary, our results provide strong evidence of a previously unsuspected role for Rho and Rac in the regulation of receptor-mediated endocytosis through coated pits. This is in marked contrast to the stimulatory effects of Rac and Rho on fluid phase endocytosis in mammalian cells (1) and xenopus oocytes (4), respectively. These findings provide further support for the mechanistic distinction between clathrin-mediated endocytosis and clathrin-independent pinocytic events (26).

References

1. Ridley, A. J. and A. Hall *Cell* **70**:401 (1992)

2. Hall, A. *Ann. Rev. Cell Biol.* **10**:31 (1994)

3. Takai, Y., T. Sasaki, K. Tanake and H. Nakanishi *Trends. Biochem. Sci.* **20**: 227 (1995)

4. Schmalzing, *et al., J. Cell Biol.* ***130***:1319 (1995)

5. Smythe, E. T.E. Redelmeier and S.L. Schmid, *Meth. Enzymol.* **219**:223 (1992)

6. Schmid, S.L. *Trends Cell Biol.* **3**:145 (1993)

7. Bokoch, G.M., B. P. Bohl and T.-H. Chuang *J. Biol. Chem.* 269:31674 (1994)

8. Ueda, T., A. Kikuchi, N. Ohgo, J. Yamamoto and Y. Takai *J Biol. Chem.* **265**:9373 (1990)

9. Lelias, J.M., *et al. Proc. Natl. Acad. Sci. (USA)* **90**:1479 (1993)

10. Scherle, P., T. Behrens, and L.M. Staudt *ibid,* :7568 (1993)

11. Aktories, K. and A. Hall *Trends Pharmacol. Sci.***10**:415 (1989)

12. Nobes, C.D. and A. Hall *Cell* **81**:53-62 (1995)

13. Malcolm. K.C., A.H. Ross, R-G. Qui, M. Symons and J.H. Exton *J. Biol. Chem.* **269**:25951 (1994)

14. Singer, W.D., H.A. Brown, G.M. Bokoch and P.C. Sternweis *J. Biol. Chem.* **270**:14944 (1995)

15. Chong, L.D., A. Traynor-Kaplan, G.M. Bokoch and M. A. Schwartz *Cell* **79**:507 (1994)

16. Hartwig, J.H., *et al., Cell* **82**:643 (1995);

17. Tolias, K.F., L.C. Cantley and C.L. Carpenter *J. Biol. Chem.* **270**:17656 (1995)

18. Bokoch, G.M., *et al. Biochem. J.* **313:** xxx (1996)

19. Kotani, K., *et al., EMBO J.* **13**:2313 (1994

20. Wennström, S., *et al., Curr. Biol.* **4**:385 (1994)

21. Claque, M.J., C. Thorpe and A.T. Jones *Febs Lett.* **367**:272 (1995)

22. Lamaze, C. and S.L. Schmid *J. Cell Biol.* **129**:47 (1995)

23. Liscovitch, M. and L.C. Cantley *Cell* **81**:659 (1995)

24. DeCamilli, P., S.D. Emr, P.S. McPherson and P. Novick *Science* **271**:1533 (1996)

25. Seaman, M.N.J., C.L. Ball and M.S. Robinson *J. Cell Biol.* **123**;1093 (1993)

26. Lamaze, C. and S. L. Schmid *Curr. Opin, Cell Biol.* **7**:573

27. Zhang, S., *et al., J. Biol. Chem.* **270**:23934 (1995)

28. Damke, H. T. Baba, A.M. van der Bliek and S.L. Schmid *J. Cell Biol.* ***131***:69 (1995)

29. Xu, X. ,D.C. Barry, J. Settleman, M.A. Schwartz and G.M. Bokoch *J. Biol. Chem.* **269**:23569 (1994)

30. Chuang, T.-H., X.Xu, U.G. Knaus, M.J. Hart and G.M. Bokoch *J. Biol. Chem.* **268**:775 (1993)

Acknowledgments

We thank Lewis Cantley for helpful discussions. This work was supported by NIH grants to SLS and GMB. CL is supported by the USAMRMC. THC is supported by a fellowship from the National Arthritis Foundation. SLS is an Established Investigator of the American Heart Association. This is TSRI Manuscript No.: 9949-CB

Figure Legends

Figure 1: Intracellular accumulation of Tfn is blocked in transiently-transfected cells expressing activated Rac and Rho mutants.

HeLa cells were grown on coverslips and transfected with expression plasmids encoding FLAG-tagged wt-Rac (**a and b**), Rac^{T17N} (**c and d**), Rac^{Q61L} **(e and f)** or HA-tagged Rho^{Q63L} **(g and h)** as previously described (27). 24 h after transfection, cells were incubated with TexasRed-conjugated Tfn (Molecular Probes,Eugene OR) for 15 min at 37°C, washed and fixed with 4% PFA for indirect immunofluroescence as described (28). Internalized Tfn accumulated in punctate endosomal structures within the cytoplasm (**b, d, f and h**). Cells expressing Rac or Rho molecules were detected by co-immunostaining with anti-FLAG (**a, c,** and **e)** or anti-HA (**g**) antibodies, respectively.

Figure 2: Regulation, by Rac and Rho, of Tfn-receptor-mediated endocytosis in a cell-free system.

Perforated A431 cells were incubated for 30 min at 37°C with K562 erythroleukemic cell cytosol, an ATP regenerating system and biotinylated-Tfn (B-Tfn). Incubations were stopped on ice and Tfn receptor-mediated endocytosis was determined by measuring the sequestration of B-Tfn from exogenously added avidin as described (5). (**a**) Assays were performed in the presence of increasing concentrations of wild-type Rac or Rho prepared from baculovirus-infected Sf9 cells as previously described (29). The efficiency of cytosol and ATP-dependent Tfn sequestration in control incubations performed in the absence of added Rho and Rac was 35 ± 8 % of total cell associated Tfn. Data shown are the average ± std. dev. from 5 independent experiments. (**b**) Assays were performed in the presence or absence of 45 nM Rac (**striped bars**) or 35 nM Rho (**solid bars**) with or without 1 μM RhoGDI, prepared as described (30). (**c**) Assays were performed in the presence of increasing concentrations of purified recombinant RhoGDI (Δ) or Ly-GDI/D4 (▲) or (**d**) in the presence of recombinant GST-C3 transferase (obtained from Dr. Simon Dillon, Tufts University) expressed and

purified from E. coli. (e) Assays were performed with or without GST-C3 transferase (5 μg/ml) and/or Rac (45 nM) as indicated. Data shown are the average ± std. dev. from 3 independent experiments.

Figure 3: Rho and RhoGDI affect clathrin coated vesicle budding in a cell-free system.

Perforated A431 cells were incubated with Tfn which had been biotinylated via a cleavable disulphide bond (BSS-Tfn) under control conditions (□) in the presence of cytosol and ATP, or in the presence of 35 nM Rho (●) or 1 μM RhoGDI (▲). Incubations were stopped on ice and the internalization of receptor-bound Tfn into sealed coated vesicles was determined by measuring the resistance of BSS-Tfn to cleavage by the small membrane impermeant reducing agent, MesNa, as previously described (5). The results show ATP- and cytosol- dependent signals obtained after subtraction of backgrounds from cells incubated at 37°C for 30 min in presence of an ATP-depleting system and in absence of cytosol.

Figure 4: Inhibition of endocytosis by Rac or Rho is not affected by either cytochalasin D or phalloidin.

Cell-free assays for Tfn sequestration were performed as described in Figure 2 in the absence (solid bars) or presence of either 10 μM phalloidin (open bars) or 10 μg/ml cytochalasin D (striped bars) without (control) or with either Rac (45 nM) or Rho (35 nM) as indicated. Control experiments, both *in vivo* and *in vitro* , in which cells were fixed, permeabilized and labeled with Texas-Red-phalloidin confirmed that cytochalasin D effectively disrupted actin stress fiber formation in the presence or absence of Rho and Rac (data not shown, but see ref. 12). The data are expressed as the percentage of total cell-associated ligand inaccessible to avidin after subtraction of background as described in Figure 3.

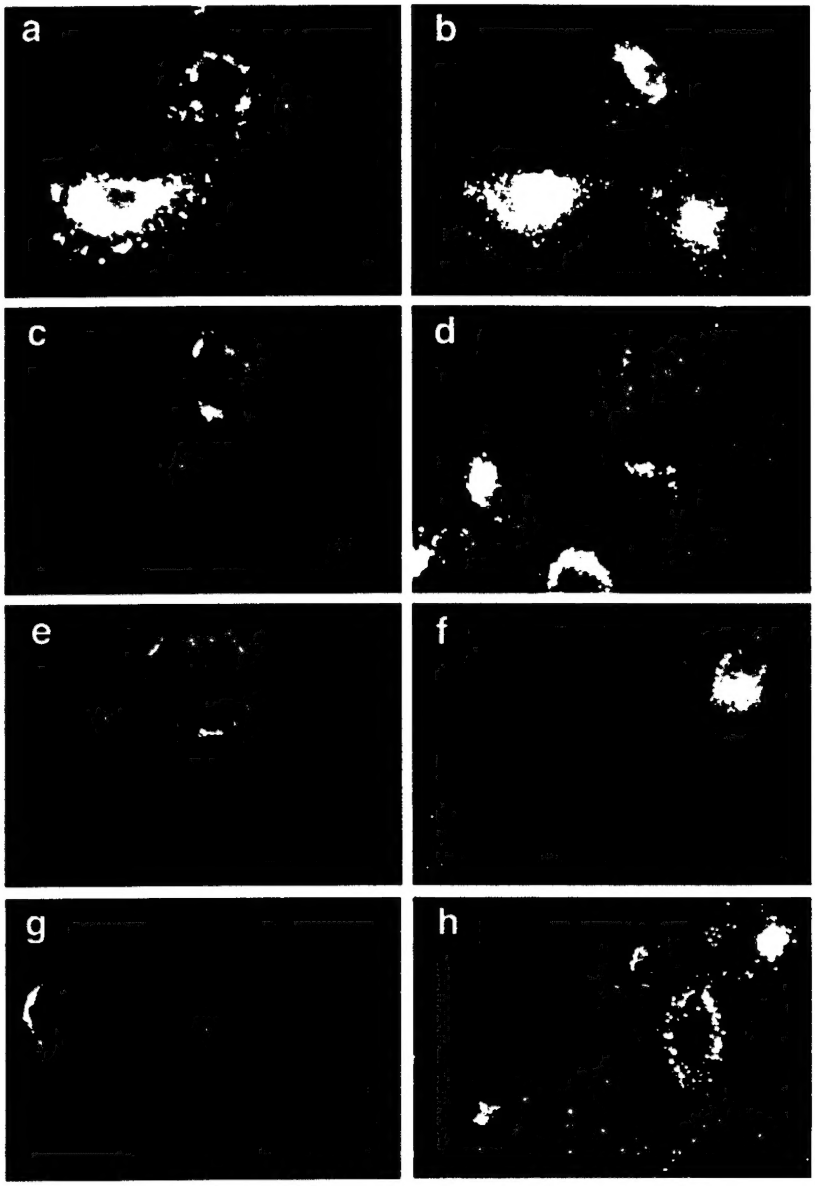

FIGURE 1
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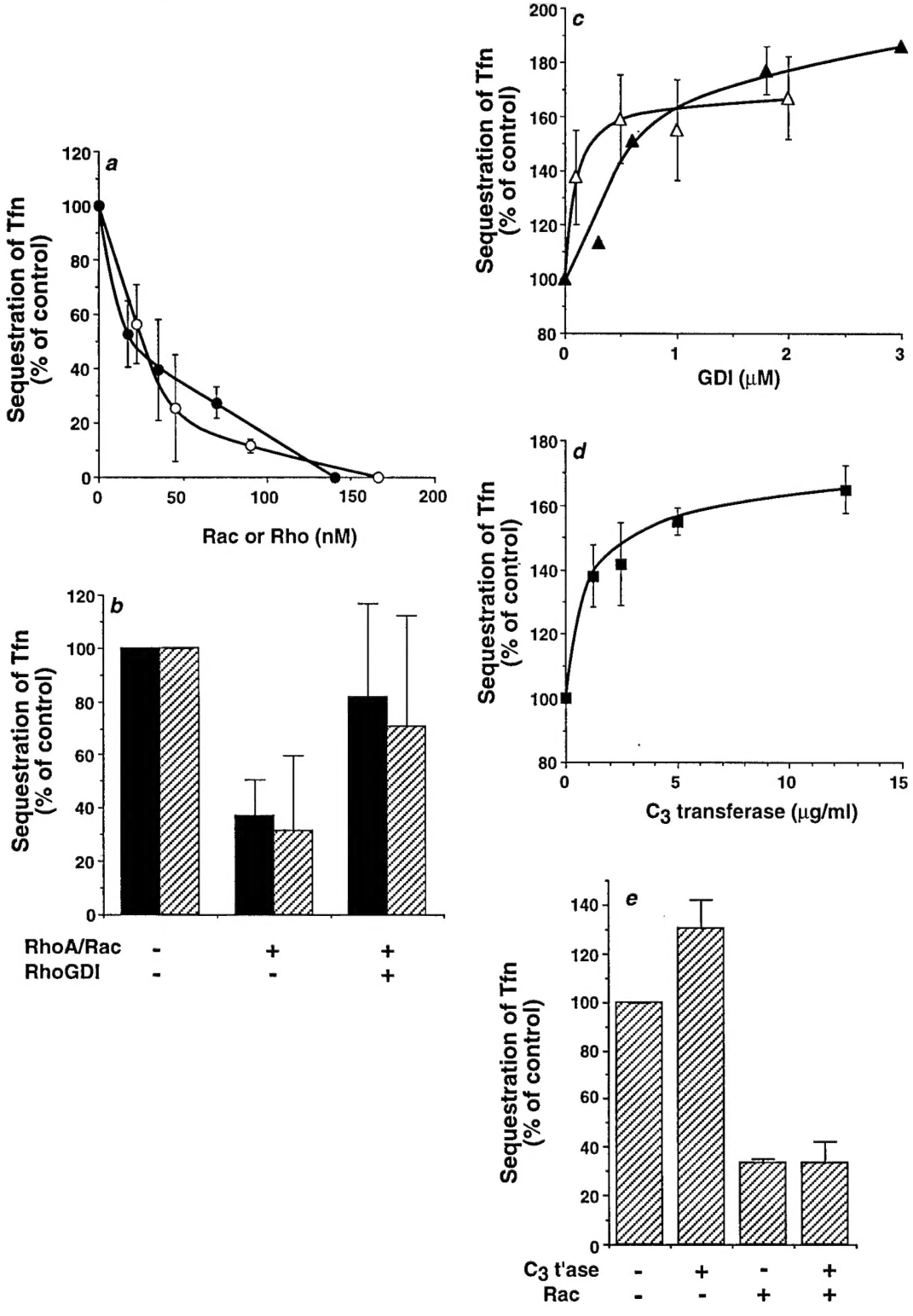

Figure 2
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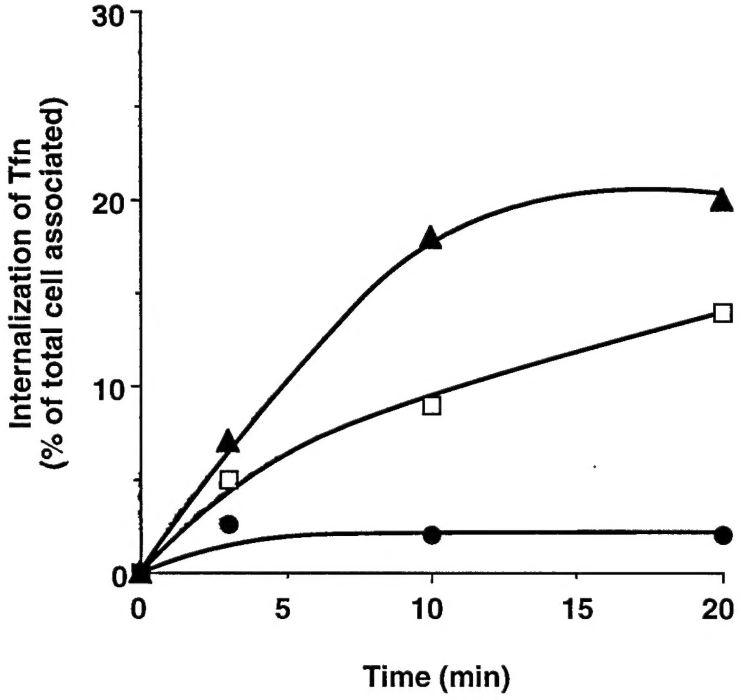

Figure 3
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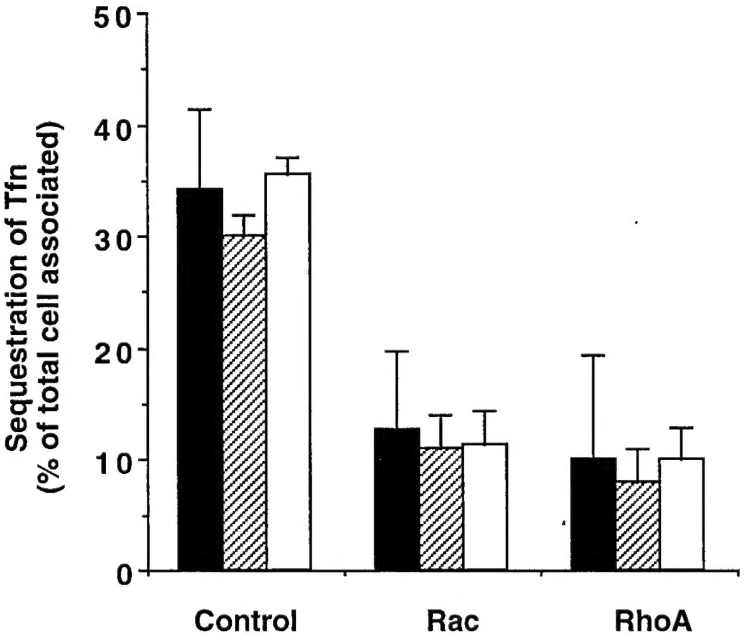

Figure 4
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